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13. ABSTRACT *(Maximum 200)*
This program consists of three projects: vascular grafts, skin micrografting and dura replacement. The vascular graft project aims to generate an off-the-shelf transplantable vascular graft for combat casualty care. We have made significant progress toward achieving a final protocol for processing vascular grafts and recent grafts implanted *in vivo* in feasibility phase studies have consistently been patent at 2 weeks. We anticipate beginning the long-term studies of 3 and 6 month duration early in year 2. The skin micrografting project aims to develop protocols for enhancing closure of full-thickness skin wounds by combining micrografting techniques with AlloDerm, a transplantable human dermis. Our studies have identified Vaseline gauze as the preferable wound dressing. Human skin proved to be unsatisfactory as a donor source for microskin pieces in our nude rat model. We are now therefore using autologous skin, which appears satisfactory as evidenced by epithelial migration from the microskin pieces. Upon verifying that we have achieved optimal grafting conditions, we will next determine the maximum expansion ratio which allows complete epithelialization of the wound. The goal of the dura replacement project is to determine whether a generic acellular matrix can function as a connective tissue scaffold for host cell repopulation and site-specific tissue regeneration. We have demonstrated in this project that XenoDerm not only serves very well as a graft, but also shows preliminary evidence that it is being remodeled into dura by the host cells which have repopulated it.

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FOREWORD

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Lawrence E. Berboom 2-5-98

PI - Signature Date

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VASCULAR GRAFT PROJECT

Introduction

The vascular graft project aims to generate an off-the-shelf, transplantable vascular graft for combat casualty care. Our efforts in the first year have been directed at developing processing methods optimized for the goat carotid artery model. This optimization is nearing completion, and grafts have been implanted in eight animals thus far. Completion of this *in vivo* study will be in the second year of this proposal. Also in the second year, we will optimize these processing methods for human vessels.

Tissue Processing

Among the steps involved in vascular graft processing are vessel procurement and protocols for decellularization, vitrification and drying of the tissue matrix.

Procurement. Harvesting and handling of tissues must be performed in a way that minimizes necrosis and liberation of cell-derived factors which may impair graft performance post-transplantation. For example, protease release secondary to cell necrosis can lead to matrix damage which can then lead to a non-specific inflammatory response and an undesirable aggressive remodeling of the matrix (1,2). Ideally, tissue is harvested fresh from a living animal and processed immediately so that cells have no time to undergo necrotic changes. Additives in the processing solution such as protease inhibitors help prevent the untoward effects of cell lysis and lysosomal release (3).

Thus far, goat carotid arteries used for analysis or implant have been procured fresh and processed immediately as described above. Therefore, we have not needed to develop a protocol to maintain cell viability post-transplantation as is done for organ do-

nation. However, once we are beyond the preliminary phase and begin processing larger numbers of vessels for use in the long-term implant portion of the study and ultimately for clinical use, practical limitations to the timing of procurement and processing will make it impossible to process fresh tissue immediately. These timing limitations make it essential to develop a 'holding step' in the process.

To this end we performed an experiment to evaluate pre-freezing as a method for storing fresh procured tissues prior to processing (Experiment 97-35; Runs #3421 and #3443). Carotid arteries were harvested from goats and the vessels were then immediately immersed in Vitrification Solution Maltodextrin (VSMD), comprised of a 75% buffered solution of maltodextrin. The vessels were then divided into two segments of equal length. One segment from each vessel was then vitrified (frozen in an amorphous or ice-free state) and stored in an -80 °C freezer. The other segment was immediately processed, which consisted of cell removal via detergents and enzymes, followed by vitrification and drying. The pre-frozen vessels were processed in a like manner after two weeks of -80 °C storage. All samples were then rehydrated and analyzed simultaneously by histology and transmission electron microscopy (TEM). Comparisons conducted by evaluators without knowledge of the treatment group demonstrated there were no significant differences between segments that were processed immediately and those that were prefrozen and stored before processing. Parameters evaluated include completeness of decellularization, basement membrane integrity, elastin morphology, collagen banding and diameter, and overall morphology. This experiment therefore demonstrates that our pre-freezing step may be implemented without danger to matrix integrity.

Decellularization. Decellularization is a critical step for the processing of transplantable tissues. The presence of cell-based antigens such as the Major Histocompatibility Groups I and II (MHC-I and II) antigens can lead to a specific inflammatory response and graft rejection (4). The LifeCell process for preserving vessels utilizes a combination of detergent and enzyme steps to fully remove cells from the arterial wall. As a first step, carotid arteries are placed in VSMD. The mechanism of action of VSMD in decellularization is unclear, but evidence suggests some polyhydroxy compounds such as glucose and its polymers promote the solubilization of some cellular proteins (5). After four hours of infiltration with VSMD, the grafts are transferred to a detergent solution with significant anti-proteolytic activity. After 24 hours in this solution, the cell cytoplasm is significantly disrupted throughout. The tissues are then transferred to a solution containing DNase and cofactors for another 24 hours to effect removal of the nuclei.

One of the problems encountered in *in vivo* studies conducted in a canine model prior to the onset of this proposal was the observation that endothelial cell (EC) coverage in transplanted grafts was incomplete. We hypothesized that the patchy EC appearance on explanted vessels was secondary to incomplete delivery of processing solutions to the center of these small diameter (2-3 mm) vessels. This idea was supported by the observation that, even though the vessels were being agitated during incubation with the decellularization solutions, there was little driving force to promote a thorough washing of their small diameter lumens.

We explored this hypothesis by modifying the decellularization protocol to enhance solution delivery throughout the lumen of the graft (Experiment 98-14, page 38; Run #3353). To accomplish this, two methods were employed. First, VSMD (the initial

decellularization solution) was initially introduced via a syringe. VSMD at its routine concentration of 75% maltodextrin is very viscous and unlikely to passively perfuse the graft. Secondly, the vessels were sutured to teflon struts which served to hold the vessel stationary within its vial while the decellularization solution was being agitated to-and-fro along its axis. This assured significant movement of the solution through the graft. Vessels strutted in this manner during processing were compared to nonstrutted vessels for completeness of decellularization and matrix integrity. These analyses were performed at five locations along the vessels' length. After staining with H&E, EC remnants were counted for each location. The fewer the number of remnants, the more effective the decellularization was deemed. While the results were equivocal with regard to whether strutting improves decellularization, we have adopted this method for processing because it improves wash through the vessel and has shown no evidence of causing matrix damage.

As another mechanism to potentially enhance donor endothelial cell removal, we explored the introduction of a specific EC removal step (Experiment 98-29, page 81; Run #3379). Three distinctly different modes of action for enhancing endothelial cell removal were investigated. First, we selected a detergent of known potency against EC's: Triton X-100. Second, 1 M sodium chloride was assessed based on its success with removing keratinocytes from their basement membrane in previous studies (6). And for a third mechanism to enhance cell removal, we utilized EDTA to chelate calcium. This divalent cation is a known cofactor for EC integrin binding to their ligands on the basement membrane (7). Integrin-ligand disruption should promote cell detachment.

In this experiment a total of 30 segments of goat carotid arteries were immersed in one of the following solutions as the first step in processing:

1) 15 mM Hepes buffer alone, pH 7.4
2) Hepes buffer plus 10 mM EDTA
3) Hepes buffer plus 10 mM EDTA plus 0.5% Triton X-100
4) Hepes buffer plus 10 mM EDTA plus 1 M NaCl
5) Hepes buffer plus 10 mM EDTA plus 0.5% Triton X-100 plus 1 M NaCl

Solutions containing the vessels were kept at 4 °C during the incubation to inhibit cell necrosis. Segments were removed for analysis after 30, 60, and 120 minutes and were then analyzed for extent of endothelial cell removal. Histology revealed that groups 4 and 5 (Hepes buffer, EDTA, NaCl, +/- TX-100) had complete endothelial cell removal after 60 minutes.

As another measure to determine whether active washing within the lumens of vascular grafts during processing could be improved, we investigated active vessel perfusion via a small roller pump (Experiment 98-33, page 94; Run #3426). After the VSMD pre-incubation, a catheter was inserted into each vessel, the vessels were immersed in the detergent solution and perfused with this solution by peristaltic pump at a flow rate of 100 ml/min. For comparison, contralateral vessels were attached to struts as described above and then placed on rotators. After the 24 hour detergent step, all segments were treated identically for the remainder of the processing and were then evaluated. Histology revealed both groups to be effectively decellularized, both with respect to EC's and SMC's. Though the pump apparatus theoretically provides the best assurance of vessel perfusion, it was cumbersome to operate – particularly in an aseptic setting. We have

therefore continued to process utilizing the strut and rotator method. We plan to devote more time and resources to the pump method when we explore human vessel decellularization.

With a satisfactory perfusion method in place, we turned our attention to establishing the optimal decellularization protocol for the initiation of *in vivo* studies (Experiment #31, page 87; Run #3386). As described above, many of the decellularization steps were transferred from protocols designed to process other tissues, and it was unclear if they were ideal for caprine vessels. We also sought to assess colder processing temperatures. Cooler temperatures, in theory, promote matrix stability and decrease the rate of potential adverse reactions, such as proteolytic and oxidative activities. Utilizing the above results and considerations, we designed the following experiment to test the decellularization effectiveness of a variety of combinations of processing steps. The experimental design is shown in Table 1.

Table 1: Decellularization Experiment

		Treatment Number												
	Processing Step	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
EC removal	1 M NaCl, 10mM EDTA, 4 °C		X	X	X	X	X	X	X		X	X	X		X
	1 M NaCl, 10mM EDTA, 20 °C									X					
VSMD Step	75 % VSMD, 20 °C	X	X							X				X	
	Isotonic (27%) VSMD, 4 °C			X	X	X					X	X			X
Detergent Step	Caprylate/CHAPS, 20 °C	X	X	X	X		X	X		X				X*	
	Caprylate/CHAPs, 4 °C					X			X		X				X
	Octyl-glucoside, 4 °C											X	X		
Enzyme Step	DNase, 37 °C	X	X	X		X	X			X				X*	
	DNase, 20 °C				X			X	X		X	X	X		X

* solutions *not* in RPMI

An 'X' in the column indicates that treatment group received the corresponding step. Most treatments received one of the four main steps; however, some treatments did not

receive the EC removal step, nor the VSMD preincubation step. In this experiment, detergents and enzymes were prepared in RPMI (with the exception of Treatment #13), a common cell culture medium. This modification was transferred from LifeCell's heart valve program which is being conducted in parallel. In that program, the addition of RPMI enhanced decellularization effectiveness. This may be due to its ability to maintain the cells in a more physiologic state until detergents and enzymes can penetrate the tissue. It is also of note that RPMI contains glucose which, as mentioned above, has been noted by us and others to enhance the solubility of some cell proteins. We also investigated the use of octyl-glucoside, a non-denaturing detergent which has been shown to render heart valves less stiff than when processed with caprylate. This is relevant in that stiffness was shown to correlate with cracking of the valves following rehydration in that study and we had observed cracking in some vascular grafts.

Vessels were processed according to these protocols and then evaluated by histology, TEM and scanning EM. Note that Treatments 10 and 14 are identical except that vessels in treatment 14 were of greater length and were intended specifically for implantation *in vivo*.

Samples processed via Treatment 8 demonstrated the best overall appearance. Assessment criteria primarily included EC or SMC remnant detection, basement membrane integrity, and the presence or absence of cracks. These results were verified in a repeat experiment (Experiment 97-37, page 11; Run 3430). Vessels processed by treatments 8 and 14 were grafted into goat carotid arteries and the results from these implants are discussed in a following section.

Freeze-Drying: After decellularization, vessels are infiltrated with the cryoprotectant, VSMD. This solution of 75% buffered maltodextrin, a polymer of glucose, significantly inhibits the diffusion and availability of water. This serves to limit waters ability to form, and migrate to, hexagonal ice crystal nucei. Tissue samples cryoprotected in this manner can be readily vitrified; that is, frozen in the amorphous or glass state. This is desirable since hexagonal ice crystal formation can mechanically disrupt and damage extracellular matrices (8). This damage can then lead to a non-specific inflammatory response and undesirable remodelling.

One problem we have encountered with this solution, however, is the occasional appearance of shallow cracking on the lumenal surface of vessels. These cracks typically only penetrate one or two elastin layers from the lumen. Interestingly, these cracks have not been observed on the adventitial surface. These cracks have been observed in processed samples, as noted above, and also in explanted grafts – usually the defect is filled with a local accumulation of fibrin and scattered cells. These defects present a nidus for platelet aggregation and activation and are thus a potential cause of graft failure.

Based on gross observations of dried material (vessels and heart valves), we believe these cracks are due to differing drying characteristics between the tissue and the residual surface coating of VSMD. Because the cryoprotectant solution is highly viscous, it is impossible to drain it completely from all surfaces. A less viscous solution would be more amenable to such removal, so we recently conducted an experiment on heart valves to test this hypothesis (Experiment 97-46, page 34). Aortas which had been incubated in a 35% solution of VSMD, and then drained of all residual, were found to not exhibit surface cracks. In contrast, the 75% VSMD controls, contained numerous cracks. Though

the 35% solution is not of sufficient strength to prevent all hexagonal ice crystal formation, it does significantly limit its overall mass. This concentration has been found to be acceptable for, and is currently employed in, the production of AlloDerm®. We currently have experiments under way to determine if it is equally efficacious in vascular grafts.

***In Vivo* Studies – The Caprine Carotid Model-Feasibility Phase**

After the production of suitable vessels, the next aim of this proposal is to test these grafts in the caprine carotid model. The protocol for this study was co-developed by us and our collaborators at the U.S. Army Institute for Surgical Research (ISR) in San Antonio. Animal procurement, implantation, and follow-up has all been conducted at the ISR. In this report, we will discuss the general results of the implants conducted to date.

Thus far, we have implanted vascular grafts in eight animals. For a summary of these results, please refer to Table 2.

Table 2: Graft Results at Two Weeks Post Implant

LIFC Goat #	*ISR Goat #*	*Implant Date*	*Days Dur'n*	*Graft Protocol*	*Control*	*Anti-plt Regimen*	*Patency*	*Explant Notes*	*Expt No*	*Run No's*	*Explant Histo.*
1	292	10/2/97	18	Tx – 14	Auto. Vein	ASA 325mg bid No Persantine	Graft no Control yes	thrombus adherent at anas-tomoses	98-31	3427	Media 50% repopulated.
2	293	10/7/97	20	Tx – 14	Auto. vein	ASA 325mg bid Persantine 25mg bid	Graft no Control no	thrombus adherent through-out	98-31	3438	Media 50% repopulated.
3	294	11/6/97	15	Tx – 8	Auto. vein	ASA 650, 1300mg qd Persantine 50,75mg bid	Graft yes Control yes	(thrombus, but very very recent) Evan's blue neg. except for radial cracks	98-37	3438	EC's present but decrease toward distal anast. Poor media repop.
4	296	11/18/97	13	Tx – 8	Auto. vein	ASA 1300mg qd Persantine 75mg bid	Graft yes Control no	Graft almost totally occluded w/ thrombus. Radial cracks. Ceph. vein remodelled	98-37	3464	As for goat 3, but fewer EC's.
5	302	11/24/97	16	Tx – 8	Auto. vein	ASA 1950mg qd Ticlopidine 250mg bid	Graft yes Control yes	No thrombus. Radial cracks.	97-41	3468	Good EC coverage most sections. Be-ginning medial repop.
6	303	11/25/97	15	Tx – 8	Auto. vein	ASA 1950mg qd Ticlopidine 250mg bid	Graft yes Control yes	No thrombus. Radial cracks.	97-41	3468	Good EC coverage most sections. Be-ginning medial repop.
7	304	12/4/97	14	Tx – 8	Auto. vein	ASA 1950mg qd Persantine 75mg bid	Graft yes Control yes	Mostly Evan's blue positive - occasional central white patch. No thrombus. Few radial cracks.	97-41	3469	Scant medial repopulation. EC's noted at anastomoses and 'M' section.
8	308	12/8/97	14	Tx – 8	Auto. vein	ASA 1950mg qd Ticlopidine 250mg bid	Graft yes Control yes	Clot present in cephalic vein graft but not completely ob-structive. Some thrombus at LifeCell graft anastomoses. Radial cracks.	97-41	3473	Scant medial repopulation. EC's noted at anastomoses and 'M' section.

Test grafts were processed according to treatment 8 discussed above, with the exception of the first two animals which received grafts from Treatment 14. The control has been the autologous cephalic vein. Implant durations varied from 13 to 20 days. As indicated, all but the first two test grafts were patent; however, there was significant thrombus associated with the third and fourth grafts. Because of the occlusions or presence of significant amounts of thrombus in this initial series of grafts, antiplatelet therapy was increased, and Ticlopidine was substituted for Persantine. All the subsequent test grafts have been widely patent.

Cell repopulation is noted in the right-most column and has been variable, both with respect to EC coverage of the lumen and SMC repopulation of the media. Evan's blue dye, which binds to albumin and cannot cross an intact EC lining, was used to delineate the extent of EC coverage and to corroborate histological findings. This dye indicated that grafts 5 and 6 were 80 to 90 percent fully endothelialized at two weeks. Histology revealed that the first two grafts were 50% repopulated with cells exhibiting a smooth muscle cell morphology.

Conclusions

We are currently undergoing an extensive analysis of variables to identify potential parameters which may significantly impact the revitalization of these grafts. Identification of these variables will ensure a more consistent pattern of repopulation. One such variable is the aforementioned surface cracking problem. This problem has been solved in aortas and we are confident a similar approach will work for these smaller arterial tissues.

Early in year 2 of this project we anticipate resolving the remaining processing issues which will lead to grafts that are repopulated with SMC and EC and do not crack. During this phase of the project we will continue to explant grafts for evaluation two weeks after implantation. Upon achieving a satisfactory processing protocol, as demonstrated by these studies, we will begin the definitive long-term studies of 3 and 6 month duration. Also in the second year we will optimize methods for the processing of human vessels.

SKIN MICROGRAFTING PROJECT

The aim of the microskin grafting project is to enhance the ability to cover extensive full-thickness skin wounds, and hence decrease patient morbidity and mortality, by developing optimal protocols for combining micrografting technologies with AlloDerm®, a transplantable human dermis.

Introduction:

Currently, the standard grafting procedure for full thickness skin injury involves the use of autologous split thickness skin grafts (STSG) (9,10). While this has been shown to be a life saving procedure, there is still a need for improvement with regard to donor site trauma and the final cosmetic and functional outcome of the wound. In full-thickness skin injuries, STSG must provide both dermal and epidermal components at the wound site. When autologous donor sites are limited, the STSG must be meshed and expanded to allow coverage of the entire wound area. This meshed configuration leaves areas of the wound uncovered by both dermis and epidermis. Epithelial cells of the grafted epidermis eventually migrate into and cover the interstices of this mesh pattern and thereby promote wound closure. However, as dermis is not a regenerative tissue, problems with scarring and contracture arise later when the grafts contract due in part to a lack of sufficient dermis.

Another approach when STSG donor sites are limited is the use of cultured epithelial autografts (CEA). This technology was the result of extensive research that established *in vitro* culture conditions necessary for the expansion of epidermal keratinocytes (11-13). Intact sheets of autologous keratinocytes can be produced from a small biopsy obtained from the patient. The production of these sheets however requires weeks of culture time. Although initial interest and use of this technology was high, as long term results became available it was evident that the

lack of dermal replacement imparts significant limitations on this approach including low overall take rates, scarring, and immature basement membrane formation leading to fragility of the epidermis (14).

An additional alternative for covering extensive burn wounds is micromeshing or microskin grafting (15-17). When autologous donor sites are limited, the available STSG can be meshed and widely expanded (generally at a ratio of 4:1 or greater) or minced by passing the tissue several times in different orientation through a standard mesher (18). While studies have shown that widely meshed autografts can eventually close a large full-thickness skin wound, these grafts a) take a long time to re-epithelialize interstices of the meshed graft, b) result in a "cobblestone" appearance at the graft site and c) often lead to debilitating scarring and contracture. Microskin grafting requires placement of many small pieces of the STSG onto the wound site. The orientation of these small pieces (epidermal side up or down) has been determined in some studies to be important for allowing migration of epithelial cells from the donor tissue onto the wound bed. This has made the application of microskin grafts very tedious, and poses one of the major limitations of this procedure. Further, these grafts are also prone to the same limitations of the meshed and expanded grafts mentioned previously. As is the case for CEA grafting, the lack of dermal replacement in these procedures presents significant limitations on the final cosmetic and functional outcome.

The problems stated above may be overcome by using the micromesh or microskin grafts as an overlay in combination with AlloDerm dermal matrix. AlloDerm preserved dermal matrix is human cadaveric skin processed to remove cells of the epidermis and dermis while leaving the structural matrix of the dermis and basement membrane complex intact. Immunohistochemical

studies have confirmed retention of basement membrane complex proteins including collagen types IV and VII, laminin and heparin sulfate proteoglycan.

It is anticipated that the "cobblestone" appearance and debilitating wound contraction exhibited in the aforementioned micromeshing procedure would be greatly reduced or eliminated by using a dermal replacement with an intact basement membrane complex.

AlloDerm dermal matrix has been used successfully for more than 2 years as a dermal replacement for full-thickness burns and to revise scarred skin tissues (19). The use of AlloDerm in these situations allows procurement of ultrathin autografts and thereby decreases the time of healing and potential complications at the donor sites. Since the autograft will be used merely as a delivery mechanism for epithelial cells, it is anticipated that the donor tissue will be very thin. Further, by using the widely expanded or micromesh techniques it is anticipated that not only will the thickness of the autograft be minimal, but the size of the donor sites may be significantly reduced as well. Epithelial cells from an overlying, meshed STSG, have been seen to migrate onto the intact basement membrane presented by AlloDerm dermal matrix. In the procedure proposed here, this should lead to the eventual loss of the overlying autograft pieces (as the epithelial cells grow under the autograft and begin to differentiate) after it has contributed the necessary epithelial cells. Loss of the overlying autograft tissue should enhance the final cosmetic outcome of the graft area by exhibiting a smooth rather than a "cobblestone" appearance.

Earlier studies performed in vitro have exhibited the formation of an epithelial cover on AlloDerm dermal matrix when donor tissue was minced and placed on the dermal graft at the air/liquid interface (at expansion ratios > 10:1). Further, these studies did not indicated a

necessity to orient pieces of the minced tissue. Epithelial cells have been seen to migrate from the donor tissue and onto the AlloDerm dermal matrix regardless of orientation.

It has been proposed that AlloDerm dermal matrix will provide a non-immunogenic dermal component for widely meshed or microskin autografts that will enhance functional performance and cosmetic appearance resulting from these two grafting procedures.

Military relevance: Burns comprise a significant percentage of casualties sustained during armed combat. A more functional skin replacement would decrease long-term morbidity and mortality in the thermally injured patient.

Body:

These studies are being carried out in collaboration with the U.S. Army Institute of Surgical Research, San Antonio, where all animal components of the work is being performed. The objective of this study is to compare the wound healing characteristics of widely meshed skin used in the presence or absence of dermal replacement. These experimental studies are designed with an initial phase in which optimal grafting conditions are defined for micromeshed skin using the nude rat model. Included in this phase are studies designed to allow familiarity with a micromeshing device (HUMECA, The Netherlands) and establishment of the most appropriate dressing for this grafting procedure. Once these parameters are established larger expansion ratios of the micromeshed skin graft will be examined.

Experimental Design: Phase I grafting studies were designed to evaluate various dressing materials that would maintain sufficient moisture to guard against desiccation of the dermal matrix while not retaining so much moisture as to cause maceration of the graft. An initial ratio of wound area to micromeshed skin of 6:1 was selected for evaluation. If a dressing is found to be successful (i.e. the dermal matrix is rapidly repopulated with host cells and

epithelial cells from the overlying micromeshed human skin migrate onto the surface of the dermal matrix) we will then move to a Phase II definitive study to examine the largest possible expansion ratio that leads to epithelialization of the entire wound area. If the initial studies do not result in repopulation of the dermal matrix or re-epithelialization of the wound from the human micromeshed skin we will examine changes to the dressing and/or a two-step grafting procedure by applying the dermal matrix 3-5 days prior to the micromeshed human skin and thereby allow repopulation and revascularization of the dermal matrix prior to application of the human skin. If this study is successful we will then move on to the Phase II study as described above. If the Phase I studies still do not result in epithelialization of the dermal matrix from the overlying micromeshed human skin we will examine the use of autologous nude rat skin as the overlying micromeshed skin. It is possible that the nude rat may not stimulate or support growth and migration of human epithelial cells. Human skin is being used primarily to allow definitive identification of the source of epithelial cells that eventually cover the wound area.

Experimental Methods: Full-thickness 4 X 6cm wounds were created on the dorsal side of each rat. AlloDerm dermal matrix was stapled in place on the open wound and covered with micromeshed human skin at a ration of 6:1. The human skin was meshed using an air driven micromeshing device from HUMECA (The Netherlands). This device cuts the skin into 3 mm^2 pieces that were then attached to an expandable transfer membrane using a biological spray adhesive. The transfer membranes are pre-folded to allow for various expansion ratios.

Initial Results: Initial studies evaluated two different dressings for the micrografting procedure, Biobrane® and Vaseline® gauze. Biobrane wound dressing (Dow Hickam Inc,

Sugarland, TX) is an adherent flexible dressing. It is composed of a semi permeable silicone membrane, bonded to a flexible nylon fabric and purified peptides derived from porcine dermal collagen. Vaseline petrolatum gauze, the second dressing evaluated, forms an occlusive layer. In these initial studies, 13 animals were covered with the Vaseline gauze dressing and 12 animals with the Biobrane dressing. Animals covered with Biobrane dressing exhibited evidence of bacterial contamination and the overall graft "take" of the dermal matrix was estimated to be < 60%. In contrast, animals covered with Vaseline gauze dressing showed no evidence of contamination and graft take rate was estimated to be > 95 %.

We also noted during these initial studies that the transfer membrane which was used to transfer micromeshed human skin to the wound surface was buckling during the first 5 days post-grafting due to contracture of the wound. This buckling caused some of the micromeshed skin pieces to be pulled away from the graft surface. These skin pieces were subsequently lost at scheduled dressing changes.

Review of histology from the initial twenty animals that received AlloDerm and micromeshed human skin, with either of the above mentioned dressings, revealed repopulation of the dermal matrix with host fibroblast but very limited epithelialization of the AlloDerm basement membrane complex. There was limited epithelialization of the dermal matrix from rat epithelium at the wound margin but little if any migration from the human microskin pieces. These results were surprising as the dermal matrix had become fully repopulated with the normal milieu of host cells and had remained intact and relatively free of desiccation.

These results indicate that a) there may be limited viability of the human skin microskin pieces, b) human epithelial cell growth and migration is not stimulated in the nude rat model or

c) the dermal matrix is inhibiting or limiting the growth and migration of epithelial cells from the overlying microskin pieces.

Based on these initial studies, we made the following changes to the grafting protocol:

A) Due to the increased bacterial contamination and decreased "take" rate when using the Biobrane dressing, we are now using the Vaseline gauze dressing on all animals.

B) The amide transfer membrane used to transfer micromeshed skin to the wound was replaced with a hand made template containing holes that correspond to placement of micromeshed skin at an expansion of 6:1. The template is removed after placement of the micromeshed skin pieces and prior to application of the dressing material. Twenty animals have been grafted using this template and the Vaseline gauze dressing. While this technique solved the problem of microskin pieces being pulled away from the surface, histological examination did not exhibit epithelial migration from the microskin pieces.

C) To determine whether the human skin might lack viability and thus account for the failure of epithelial migration to occur from the microskin pieces we investigated skin viability and storage conditions. Human skin for this procedure was generally obtained the day before the animal surgery due to the timing of the clinical surgical schedule. The skin was stored at 4 degrees centigrade in normal saline. We have recently grafted animals with skin that was stored in saline (n=5) as described above or stored at 37 degrees centigrade in a cell culture support medium containing 10% bovine serum (n=5). Animals were grafted with human skin alone, in the absence of the dermal matrix. A portion of each skin sample was also transferred to normal cell culture to examine whether cells could be

cultured from the skin in standard in-vitro conditions. At 14 days post grafting, skin samples placed into *in vitro* culture conditions exhibited only limited growth. Histological examination of biopsies from the grafted animals did not exhibit epithelial migration from the human microskin pieces. These data cost doubt on the viability of the human skin as used in the early grafting studies.

D) We are currently following up on this study by examining the use of autologous rat skin removed during creation of the wound. Five animals have been grafted with autologous rat microskin pieces. These skin pieces were produced in the same manner as described for the human skin. While the use of autologous skin will not allow definitive identification of the source of epithelial cells covering the wound, it is on the other hand directly in line with the anticipated human clinical application of this procedure, which is to use autologous microskin pieces.

Conclusions:

We have demonstrated that Vaseline gauze is the dressing of choice for these microskin grafting procedures. Our studies have also demonstrated that human skin, as it was used in the initial grafts, is unsatisfactory. This may be because of poor viability or because it is incompatible with the nude rat model. Preliminary data suggest that use of autologous skin will solve this problem. Once we have defined the optimal grafting conditions, which we anticipate we will achieve early in year 2, we will implement the definitive study in which we will determine the largest possible expansion ratio which allows complete epithelialization of the wound.

DURA REPLACEMENT PROJECT

The goal of the dura replacement project is to determine whether a generic acellular matrix can function as a connective tissue scaffold by providing a compatible biological framework for host cell repopulation and site-specific tissue regeneration in the replacement of dura mater. During year 1 bilateral duraplasty was performed in 12 minipigs, with AlloDerm used as dura replacement on one side and pericranial tissue on the contralateral side. Specimens were obtained for evaluation from four pigs at both the 1 and 3 month time points following implantation. The final four pigs will be evaluated at 6 months, which will occur in year 2.

Introduction

The dura mater, a layer of vascularized dense connective tissue, is a vital neurological membrane which serves to protect and maintain the central nervous system. This membrane may be damaged due to trauma, during tumor removal or other neurosurgical and reconstructive procedures. This can result in hemorrhage, cerebrospinal fluid leakage, infection and even seizures. Because of the severity of the symptoms induced by disruption of the dural integrity and its essential role in the nervous system, there has been a long and extensive search for viable dural substitutes. Materials used for this purpose have ranged from metal foils (20) to plastics (21), xenographic materials (22) and autologous (23) and allograft tissues (24). None of these materials have proved to be fully satisfactory. We therefore are examining in a minipig model the utility of XenoDerm, an acellular dermal matrix processed from porcine skin, to serve as a dura replacement. XenoDerm is the porcine equivalent of AlloDerm®, which is processed from human cadaver skin and has already been shown to be efficacious in the

treatment of full thickness burns (19). It has also been used for soft tissue augmentation in plastic surgery (25) and has the potential to be useful in tissue repair and regeneration in diverse situations. XenoDerm rather than AlloDerm is being used in this study in order to test the material in an allogenic situation and thereby more accurately simulate the clinical condition.

Experimental Methods and Results

This project is being carried out in collaboration with Mt. Sinai Medical Center, New York, where the surgical components of the study are performed. In twelve minipigs we have performed bilateral craniotomies, thereby exposing the dura mater. Circular sections of dura measuring approximately 2.5cm in diameter were then excised. On one side XenoDerm was used as a dural replacement patch. A matching reconstruction was performed on the contralateral side using autologous pericranial tissue. The pigs were then allowed to recover. At each of the one, three and six month time points, four pigs are scheduled to be killed and the dural replacements examined. The one and three month evaluations were completed during year 1 of this project and the six month evaluation will take place early in year 2.

Results

There were no post-operative complications and all pigs survived to their scheduled date of euthanasia. The XenoDerm performed very well as a dural replacement in all cases. Macroscopic evaluation demonstrated there were no adhesions to the brain and only modest adhesions to the cranium at the margins of the craniotomy. There was no evidence of inflammation or cerebral spinal fluid leak. All grafts were intact, retained their original dimensions and resembled the surrounding dura.

The grafts formed from pericranial tissue had become thickened at 3 months as compared with its thickness when implanted. The pericranial tissue grafts also contained bony outgrowths that were tightly adherent to the graft surface. Adherence to the brain was minimal.

Microscopic evaluation at 1 month demonstrated repopulation of XenoDerm grafts with fibroblasts and evidence of neovascularization. Immunohistochemical analysis using a collagen type IV antibody exhibited retention of the basement membrane complex of XenoDerm grafts and evidence of cell repopulation in areas corresponding to pre-existing vascular channels of the dermal matrix. The pericranial tissue grafts exhibited a greater number of infiltrating cells at 1 month as compared with the XenoDerm grafts.

At three months, some areas in the XenoDerm grafts had taken on a dense connective tissue appearance which was similar to that of the surrounding dura and contrasts with the more loose connective tissue conformation that is typical of the dermis from which this graft is derived. This suggests an early stage of remodeling, in which XenoDerm is being repopulated by host cells and converted to dura. We anticipate confirmation that remodeling is indeed occurring when data from the six month time point become available in year 2. In the autologous tissue grafts at three months, hypercellularity continues to be exhibited. Trichrome stain also reveals a decrease in mature collagen as compared to surrounding normal dura.

Conclusion

This study is nearing completion. Data at 1 and 3 months demonstrate that XenoDerm holds strong promise as a replacement for dura. If results at 6 months

confirm the suggestion that XenoDerm is being remodeled into dura, a strong case can be made for the potential of XenoDerm to serve as a generic acellular matrix which can function as a connective tissue scaffold by providing a compatible biological framework for host cell repopulation and site-specific tissue regeneration.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 16 Nov 00

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-97-2-7004. Request the limited distribution statement for Accession Document Numbers ADB239008, ADB243192, and ADB258557 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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